# *Formosa haliotis* sp. nov., a brown-alga-degrading bacterium isolated from the gut of the abalone *Haliotis gigantea*

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Four brown-alga-degrading, Gram-stain-negative, aerobic, non-flagellated, gliding and rod-shaped bacteria, designated LMG 28520$^T$, LMG 28521, LMG 28522 and LMG 28523, were isolated from the gut of the abalone *Haliotis gigantea* obtained in Japan. The four isolates had identical random amplified polymorphic DNA patterns and grew optimally at 25 °C, at pH 6.0–9.0 and in the presence of 1.0–4.0 % (w/v) NaCl. Phylogenetic trees based on 16S rRNA gene sequences placed the isolates in the genus *Formosa* with *Formosa algae* and *Formosa arctica* as closest neighbours. LMG 28520$^T$ and LMG 28522 showed 100 % DNA–DNA relatedness to each other, 16–17 % towards *F. algae* LMG 28216$^T$ and 17–20 % towards *F. arctica* LMG 28318$^T$; they could be differentiated phenotypically from these established species. The predominant fatty acids of isolates LMG 28520$^T$ and LMG 28522 were summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), iso-$C_{15:1}$ G and iso-$C_{15:0}$. Isolate LMG 28520$^T$ contained menaquinone-6 (MK-6) as the major respiratory quinone and phosphatidylethanolamine, two unknown aminolipids and an unknown lipid as the major polar lipids. The DNA G+C content was 34.4 mol% for LMG 28520$^T$ and 35.5 mol% for LMG 28522. On the basis of their phylogenetic and genetic distinctiveness, and differential phenotypic properties, the four isolates are considered to represent a novel species of the genus *Formosa*, for which the name *Formosa haliotis* sp. nov. is proposed. The type strain is LMG 28520$^T$ (=NBRC 111189$^T$).

Abbreviation: RAPD, random amplified polymorphic DNA.

The GenBank/EMBL accession numbers for the 16S rRNA gene sequences of LMG 28520$^T$, LMG 28521, LMG 28522 and LMG 28523 are LC005522, AB542074, AB542075 and AB542076, respectively.

Four supplementary figures are available with the online Supplementary Material.

The genus *Formosa*, a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes* (Bernardet, 2011), was first proposed by Ivanova *et al.* (2004) with *Formosa algae* as the sole recognized species. Subsequently, four additional species of the genus *Formosa*, *Formosa agariphila* (Nedashkovskaya *et al.*, 2006), *Formosa spongicola* (Yoon & Oh, 2011), *Formosa undariae* (Park *et al.*, 2013) and *Formosa arctica* (Kwon *et al.*, 2014), were validly named. In this study, we

report on four isolates with brown-alga-degrading activity, obtained from the gut of the abalone *Haliotis gigantea*, as representatives of a novel species of the genus *Formosa* based on a polyphasic characterization.

Isolates LMG 28520$^T$ and LMG 28521 were obtained from the gut of the abalone *Haliotis gigantea* collected at an abalone hatchery (Owase, Mie, Japan) in July 2009. Isolates LMG 28522 and LMG 28523 were obtained from the same abalone species and the same location in August 2009. These four isolates were obtained by the dilution plating technique through cultivation at 25 °C on marine agar (MA; BD Difco) containing 0.5 % alginate (YPD medium; Tanaka *et al.*, 2003). Bacterial ability to degrade the algae tissues was tested using the method described by Sawabe *et al.* (2000). Briefly, algae tissue was cut in pieces of 1 × 4 cm, and put into 18 mm test tubes including 5 ml of artificial seawater. After autoclaving the test tubes, the four isolates were each inoculated into one tube that was then incubated for 2 weeks at 20 °C. After 2 weeks of incubation, the four isolates completely broke down the algae tissue.

*F. algae* LMG 28216$^T$ and *F. arctica* LMG 28318$^T$ were used as reference strains for DNA–DNA hybridizations, analyses of fatty acids and phenotypic characterization. *F. algae* LMG 28216$^T$ was also included in random amplified polymorphic DNA (RAPD) analysis. Genomic DNA for RAPD, DNA–DNA hybridizations and DNA G+C content determination was extracted using a modification (Cleenwerck *et al.*, 2002) of the method described by Wilson (1987).

RAPD analyses of *F. algae* LMG 28216$^T$ and the four new isolates were performed as described by Williams *et al.* (1990), using primers 1 (5′-GGTGCGGGAA-3′), 2 (5′-GTTTCGCTCC-3′), RAPD-270 and RAPD-272 (Mahenthiralingam *et al.*, 1996). For each primer, the isolates showed the same RAPD pattern (Fig. S1, available in the online Supplementary Material), which indicates that they are probably multiple isolates of the same strain.

The 16S rRNA gene of the four isolates was amplified by PCR and sequenced using the universal primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1512R (5′-ACGGTTACCTTGTTACGACTT-3′). Pairwise sequence similarities were calculated using the BioNumerics v7.0 software package (Applied Maths). Alignment and phylogenetic analysis were performed using the MEGA 5 software (Tamura *et al.*, 2011). A phylogenetic tree was reconstructed based on nearly complete 16S rRNA gene sequences (1450 nt) using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. The isolates showed identical 16S rRNA gene sequences and exhibited highest 16S rRNA gene sequence similarity towards the type strains of *F. algae* LMG 28216$^T$ (98.1 %) and *F. arctica* IMCC 9485$^T$ (97.1 %), and less than 96.9 % towards the type strains of other species of the genus *Formosa*. The maximum-parsimony and maximum-likelihood trees (Figs S2 and S3) showed essentially the same topology as the neighbour-joining tree (Fig. 1), and placed the isolates in the genus *Formosa*.

DNA–DNA hybridizations were performed between isolates LMG 28520$^T$ and LMG 28522 and the type strains of their nearest phylogenetic neighbours (*F. algae* LMG 28216$^T$ and *F. arctica* LMG 28318$^T$). Hybridizations were performed under stringent conditions in a solution containing 50 % (v/v) formamide at 35 °C, i.e. the $T_{0R}$ + 6.1 °C, using a modified version of the microplate method described by Ezaki *et al.* (1989) (Goris *et al.*, 1998). The value of $T_{0R}$ was determined using the following equation: 0.51 × average % G+C content + 47 °C − 36 °C (correction for 50 % formamide), with the average % G+C content being 36 mol%. For every DNA pair, reciprocal reactions (e.g. $A \times B$ and $B \times A$) were carried out, and the variation observed between them was generally within the limits of this method (mean variation of ± 7 %). The experiments were performed in quadruplicate and repeated twice. Per DNA pair, the presented DNA–DNA hybridization relatedness value is the mean of the reciprocal values. Isolates LMG 28520$^T$ and LMG 28522 exhibited mean DNA–DNA relatedness values of 100 (± 12.5) % among each other, 16 (± 4.0) to 17 (± 0.5) % towards *F. algae* LMG 28216$^T$ and 17 (± 5.0) to 20 (± 7.0) % towards *F. arctica* LMG 28318$^T$, indicating that the isolates do not belong to these formally named species of the genus *Formosa* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The DNA G+C content of isolates LMG 28520$^T$ and LMG 28522 was determined with the enzyme degradation method described by Mesbah *et al.* (1989). The nucleoside mixture was separated by HPLC using a Symmetry Shield C8 column (Waters) at 37 °C using 0.02 M $(NH_4)H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. The DNA G+C content was 34.4 mol% for isolate LMG 28520$^T$ and 35.5 mol% for isolate LMG 28522, values that are similar to those reported for *F. algae* (Ivanova *et al.*, 2004), *F. agariphila* (Nedashkovskaya *et al.*, 2006), *F. spongicola* (Yoon & Oh, 2011), *F. undariae* (Park *et al.*, 2013) and *F. arctica* (Kwon *et al.*, 2014).

The whole-cell fatty acid methyl ester composition was determined for isolates LMG 28520$^T$, LMG 28522, *F. algae* LMG 28216$^T$ and *F. arctica* LMG 28318$^T$ using a 6890N gas chromatograph (Agilent Technologies). Cultivation of the strains, harvesting of the cells, fatty acid extraction and analysis of the fatty acid methyl esters were performed according to the recommendations of the Sherlock Microbial Identification System (MIDI). Fatty acids were extracted from cultures grown on MA for 3 days at 25 °C under aerobic conditions. They were identified using the TSBA identification library version 5.0. The fatty acid contents obtained for *F. algae* LMG 28216$^T$ and *F. arctica* LMG 28318$^T$ (Table 1) corresponded largely with previous results (Park *et al.*, 2013; Kwon *et al.*, 2014). The fatty acid contents of the new isolates showed similarities to those of the related species *F. algae* and *F. arctica*, but also differed in the amounts of certain components (Table 1). The predominant fatty acids of isolates

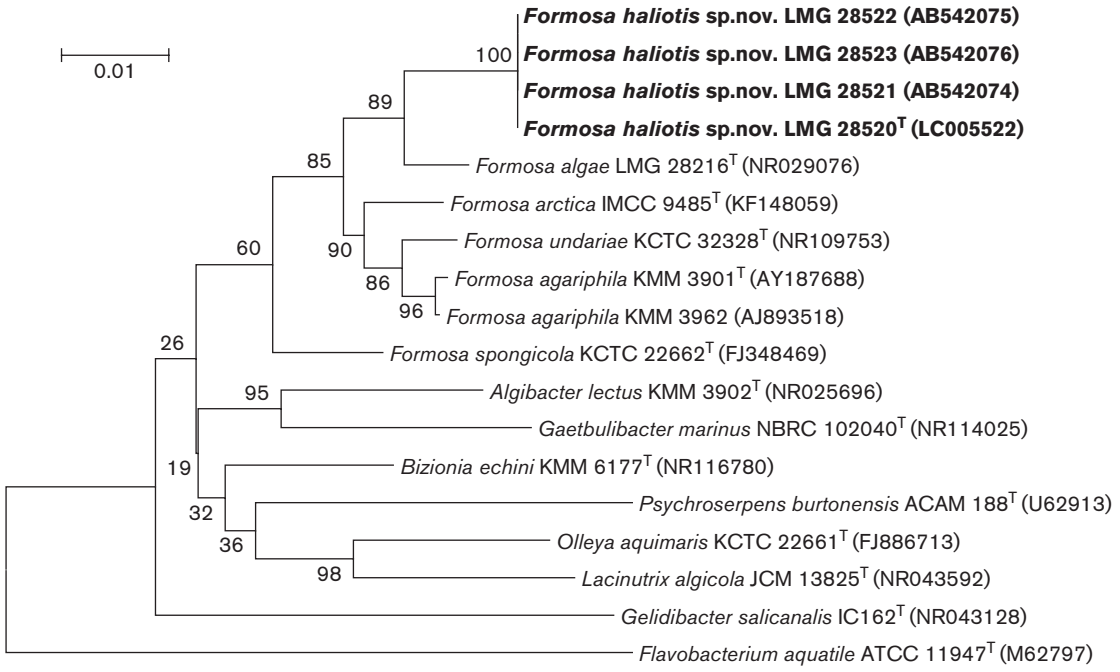


**Fig. 1.** Phylogenetic tree, based on the neighbour-joining method, showing the relationship between isolates LMG 28520$^T$, LMG 28521, LMG 28522 and LMG 28523 and representative members of the family *Flavobacteriaceae*. Numbers at nodes indicate levels of bootstrap support (%) based on a dataset of 1000 resamplings. Bar, 0.01 substitutions per nucleotide position.

LMG 28520$^T$ and LMG 28522 (>10 % of the total fatty acid content) were summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, 20.3–21.3 %), iso-$C_{15:1}$ G (11.6 %) and iso-$C_{15:0}$ (10.9–11.3 %).

Analysis of respiratory quinones was carried out by the Identification Service of DSMZ (Braunschweig, Germany). Respiratory quinones were extracted from lyophilized cells of isolate LMG 28520$^T$ and *F. algae* LMG 28216$^T$ using the two-stage method described by Tindall (1990a, b). Respiratory lipoquinones were separated into their different classes by TLC and further analysed using an LDC Analytical HPLC system (Thermo Separation Products) fitted with a reversed-phase column (125 × 2 mm, 3 µm, RP18; Macherey-Nagel) and methanol/heptane (9: 1, v/v) as the eluent. Respiratory lipoquinones were detected by UV absorbance at 269 nm. For both strains, the predominant lipoquinone was menaquinone-6 (MK-6), which is in line with previous observations for species of the genus *Formosa* (Nedashkovskaya *et al.*, 2006; Yoon & Oh, 2011; Park *et al.*, 2013; Kwon *et al.*, 2014).

Polar lipids of isolate LMG 28520$^T$ were extracted from lyophilized bacterial cells and examined using two-dimensional TLC followed by detection with the reagents molybdatophosphoric acid, ninhydrin, molybdenum blue, α-naphthol and Dragendorff's reagent (Minnikin *et al.*, 1984). The polar lipids detected for isolate LMG 28520$^T$ were phosphatidylethanolamine, two unknown aminolipids and an unknown lipid (Fig. S4), which is similar to the polar lipid profiles of other members of the genus *Formosa* (Park *et al.*, 2013; Kwon *et al.*, 2014; Shakeela *et al.*, 2015).

Cell morphology was examined by light microscopy (Eclipse 50i system; Nikon). Pigments were extracted in acetone/methanol (7: 2, v/v) and the absorption spectrum between 300 and 700 nm was determined with an Infinit 200 Pro UV–visible spectrophotometer (Tecan). Gliding motility was investigated as described by Bowman (2000). Growth under anaerobic conditions was determined after incubation for 7 days in an anaerobic jar system (MGC) with AnaeroPack (MGC) in marine broth 2216 (MB; BD Difco). Growth at 4, 10, 20, 25, 30, 35, 37 and 40 °C was measured in MB. The pH range for growth was investigated in MB adjusted to pH 4–12 by using Good's buffers (Kishida chemical). Growth in the absence of NaCl and in the presence of 1, 2, 3, 4, 5, 6 and 8 % (w/v) NaCl was investigated in yeast-peptone (1 and 5 %, w/v, respectively) broth. Catalase activity was determined by the production of oxygen bubbles after mixing cells with 3 % (v/v) $H_2O_2$. Oxidase activity was determined by oxidation of 1 % (w/v) *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine solution (Wako). Hydrolysis of alginate was tested on YPD medium as described by Tanaka *et al.* (2003). Hydrolysis of aesculin, starch, gelatin, urea, casein, chitin, cellulose and DNA was investigated using MA containing 1 % (w/v) of the substrate. Hydrolysis of Tween 80 and

**Table 1.** Cellular fatty acid contents (%) of strains LMG 28520$^T$ and LMG 28522 and their phylogenetically closest species.

Strains: 1, LMG 28520$^T$; 2, LMG 28522; 3, *F. algae* LMG 28216$^T$; 4, *F. arctica* LMG 28318$^T$. Fatty acids present in trace amounts (<1.0 %) in all strains are not shown. ND, Not detected; TR, trace amount (<1.0 %). All data were generated in the frame of this study. Cultivation conditions prior to fatty acid extraction were identical for all strains (MA, 3 days).

| Fatty acid | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| $C_{12:0}$ | ND | 1.9 | ND | TR |
| $C_{16:0}$ | 2.3 | 2.6 | TR | TR |
| iso-$C_{14:0}$ | 1.6 | 1.7 | ND | 1.9 |
| iso-$C_{15:0}$ | 11.3 | 10.9 | 18.8 | 18.3 |
| iso-$C_{15:1}$ G | 11.6 | 11.6 | 13.8 | 10.0 |
| iso-$C_{16:0}$ | 1.8 | 1.7 | 1.1 | 1.0 |
| iso-$C_{16:1}$ H | 3.2 | 2.9 | TR | 2.5 |
| anteiso-$C_{15:0}$ | 4.7 | 4.6 | 5.7 | 10.1 |
| anteiso-$C_{15:1}$ A | 2.4 | 2.2 | 2.0 | 2.1 |
| $C_{15:0}$ 2-OH | 1.4 | 1.5 | 2.8 | 2.0 |
| $C_{15:0}$ 3-OH | 2.4 | 2.4 | 3.0 | ND |
| $C_{16:0}$ 3-OH | 2.4 | 2.5 | TR | TR |
| $C_{17:0}$ 2-OH | TR | TR | 1.2 | 2.6 |
| iso-$C_{15:0}$ 3-OH | 4.8 | 5.1 | 9.8 | 9.5 |
| iso-$C_{16:0}$ 3-OH | 7.0 | 5.9 | 4.4 | 7.8 |
| iso-$C_{17:0}$ 3-OH | 4.3 | 3.9 | 8.5 | 7.9 |
| $C_{15:1}\omega6c$ | 9.3 | 8.8 | 8.6 | 3.4 |
| $C_{17:1}\omega6c$ | 2.9 | 2.8 | 5.6 | 2.5 |
| $C_{17:1}\omega8c$ | TR | TR | 2.0 | ND |
| iso-$C_{17:1}\omega9c$ | 1.5 | 1.5 | TR | 1.3 |
| anteiso-$C_{17:1}\omega9c$ | ND | ND | ND | 1.6 |
| 10-methyl $C_{18:0}$ (TSBA) | TR | TR | 1.3 | ND |
| Summed feature 3* | 20.3 | 21.3 | 5.7 | 8.9 |

*iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ (TSBA identification library v5.0); $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ (TSBA environmental aerobic bacteria library v6.1).

nitrate reduction was investigated as described by Lányí (1987) with the modification that artificial seawater was used for preparation of the media. $H_2S$ production was tested according to Bruns *et al.* (2001). Hydrolysis of acetoin was tested using VP reagents (BD Difco). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann & Baumann (1981). Acid production from carbohydrates was tested according to Leifson (1963). Susceptibility to antibiotics was tested on MA plates using antibiotic discs (BD Difco) containing (mg per disc unless otherwise stated): chloramphenicol (30), erythromycin (15), rifampicin (5), vancomycin (30), penicillin G (10), gentamicin (30), kanamycin (30), ampicillin (10), tetracycline (30), neomycin (30), polymyxin B (300 U) and streptomycin (50). Enzyme activities were determined, after incubation for 8 h at 25 °C, by using the API ZYM system (bioMérieux). Morphological, physiological and biochemical properties of the isolates are given in the species description and in Table 2. The isolates could be differentiated from their closest phylogenetic relatives (*F. algae* and *F. arctica*) based on their ability to utilize L-arabinose, citrate, cellobiose (differentiation from *F. algae*) and D-galactose (differentiation from *F. arctica*); their ability to produce β-galactosidase, α-glucosidase, β-glucuronidase and α-fucosidase; their ability to reduce nitrate (differentiation from *F. arctica*); and their inability to hydrolyse urea (differentiation from *F. algae*).

In conclusion, the phenotypic, chemotaxonomic, phylogenetic and genetic data generated for isolates LMG 28520$^T$, LMG 28521, LMG 28522 and LMG 28523 demonstrate that they represent a novel species of the genus *Formosa*, for which we propose the name *Formosa haliotis* sp. nov.

## Description of *Formosa haliotis* sp. nov.

*Formosa haliotis* (ha.li.o′tis. N.L. gen. n. *haliotis* named after the scientific name of the abalone *Haliotis*).

Cells are Gram-stain-negative, non-flagellated, gliding and rod-shaped, approximately 0.5–0.8 mm in diameter and 0.8–2.0 mm in length. Colonies are 1.0 mm in diameter after incubation for 3 days at 25 °C on MA. Cells contain carotenoid pigments with maximum absorption at 451 nm. Growth occurs from 10 to 35 °C, but not at 37 or 40 °C, and only weakly at 4 °C. Optimal growth temperature is 25 °C. Optimal pH for growth is between 6 and 9. Growth occurs in the presence of 1–8 % (w/v) NaCl with an optimum of approximately 1.0–4.0 % (w/v) NaCl. Anaerobic growth does not occur. Catalase- and oxidase-positive. Nitrate is reduced to nitrite. $H_2S$ and acetoin are not produced. Alginate, aesculin, starch and gelatin are hydrolysed, but not urea, casein, chitin, cellulose, DNA or Tween 80. L-Arabinose, citrate, cellobiose, D-galactose, D-glucose, D-mannose, D-xylose, L-rhamnose, malate, fumarate and malonate are utilized as carbon and energy sources, but not sucrose, D-fructose, maltose, D-fucose, raffinose, L-sorbose, D-adonitol, glycerol, dulcitol, inositol, D-sorbitol, acetate, mannitol, formate or amygdalin. Acid is produced from D-glucose, maltose, D-xylose, D-galactose, L-arabinose, cellobiose, L-rhamnose and alginate. Susceptible to chloramphenicol, erythromycin, rifampicin, vancomycin and penicillin G, but not to gentamicin, kanamycin, ampicillin, tetracycline, neomycin, polymyxin B or streptomycin. In assays with the API ZYM system, β-galactosidase, α-glucosidase, β-glucuronidase, α-fucosidase, alkaline phosphatase, leucine arylamidase, acid phosphatase, valine arylamidase and naphthol-AS-BI-phosphohydrolase activities are present, but not β-glucosidase, *N*-acetyl-β-glucosaminidase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-galactosidase, α-mannosidase or α-chymotrypsin. The predominant fatty acids are summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), iso-$C_{15:1}$ G and iso-$C_{15:0}$. The respiratory quinone detected is MK-6. The major polar lipids are

**Table 2.** Differential characteristics between *Formosa haliotis* sp. nov. and the type strains of recognized species of the genus *Formosa*

Taxa: 1, Formosa haliotis sp. nov. (LMG 28520$^T$, LMG 28521, LMG 28522, LMG 28523); 2, *F. algae* LMG 28216$^T$; 3, *F. arctica* IMCC 9485$^T$; 4, *F. agariphila* KMM 3901$^T$; 5, *F. spongicola* KCTC 22662$^T$; 6, *F. undariae* KCTC 32328$^T$. Data in columns 1 and 2 were generated in the frame of this study. Data in column 3 were taken from Kwon *et al.* (2014), except requirement of $Mg^{2+}$ for growth, hydrolysis of aesculin and gelatin, and utilization of carbon sources, which were generated in the frame of this study; data in columns 4, 5 and 6 were taken from Park *et al.* (2013). +, Positive reaction; −, negative reaction; w, weakly positive reaction. All strains were susceptible to chloramphenicol and positive for catalase and oxidase activities, and acid production from D-glucose, maltose and D-xylose. All strains were resistant to gentamicin and kanamycin, and negative for $H_2S$ production and hydrolysis of casein.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Gliding motility | + | + | + | + | + | − |
| Growth at 4 °C | w | w | − | + | − | + |
| NaCl range (%) for growth | 1–8 | 0–8 | 0.5–5 | 1–8 | 1–5 | 0–9 |
| Requirement of $Mg^{2+}$ for growth | − | − | − | − | − | + |
| Nitrate reduction | + | + | − | − | + | − |
| Hydrolysis of: | | | | | | |
| Tween 80 | − | − | − | − | − | + |
| Urea | − | + | − | − | − | − |
| Aesculin | + | + | + | + | − | + |
| Gelatin | + | + | + | + | + | − |
| Starch | + | −* | + | − | − | + |
| Utilization of: | | | | | | |
| L-Arabinose | + | − | − | − | − | − |
| Sucrose | − | − | − | +† | − | − |
| Citrate | + | − | − | − | − | − |
| Cellobiose | + | − | + | − | − | + |
| D-Fructose | − | + | + | + | − | + |
| D-Galactose | + | +* | − | + | − | + |
| D-Glucose | + | + | + | + | − | + |
| Maltose | − | + | + | + | − | + |
| D-Mannose | + | + | + | + | − | + |
| D-Xylose | + | + | + | − | − | + |
| Susceptibility to: | | | | | | |
| Ampicillin | − | − | + | + | − | − |
| Tetracycline | − | − | + | − | − | − |
| Enzyme activity (API ZYM): | | | | | | |
| $\beta$-Galactosidase | + | − | − | w | − | − |
| $\alpha$-Glucosidase | + | − | − | − | − | + |
| $\beta$-Glucosidase | − | − | − | − | − | + |
| *N*-Acetyl-$\beta$-glucosaminidase | − | + | − | + | − | − |
| $\beta$-Glucuronidase | + | − | − | − | − | − |
| $\alpha$-Fucosidase | + | − | − | − | − | − |
| DNA G+C content (mol%) | 34.4‡ | 34.0 | 37.6 | 35.9 | 36.0 | 37.3 |

*Results not corresponding to those obtained by Park *et al.* (2013).
†Data taken from Nedashkovskaya *et al.* (2006).
‡Data for the type strain.

phosphatidylethanolamine, two unknown aminolipids and an unknown lipid.

The type strain, LMG 28520$^T$ (=NBRC 111189$^T$), was isolated from the gut of the abalone *Haliotis gigantea* harvested in Owase, Mie, Japan. The DNA G+C content of the type strain is 34.4 mol%. LMG 28521, LMG 28522 and LMG 28523 are additional strains of the species.

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